CHAPTER 6

Discussion and Future Perspectives

Summary

Cancer and ageing are two major issues for the current society. One reason is that cancer occurrence increases with age, and over the last century, we have noticed an increase in the lifespan of people. On a cellular level, cancer and aged cells do not share striking similarity. Indeed, while cancer cells actively proliferate, aged cells are associated with a non-proliferating state known as senescence. However, genome instability is increased in both cancer and aged cells. Genome instability is often associated with mutations, loss of heterozygosity, duplications, translocations and sister chromatid exchanges (SCEs). SCEs are used as marker to measure genome instability, however little is known about the mechanism and types of DNA lesions that lead to their formation.

A significant contribution to the field we have made is the development of Strand-seq to be able to measure and map spontaneous SCEs genome-wide in a single yeast cell. Our study also contributes to a better understanding of the mechanisms behind the formation of spontaneous SCEs. While it is commonly thought that SCEs are products of double-strand break (DSB) repair, we show that this is not the major pathway of generating spontaneous SCEs. Here, I will further discuss what other mechanisms could lead to the formation of SCEs and how we could test those hypotheses. In addition to the data obtained during my PhD, one major part of my work was to adapt Strand-seq for use with yeast cells to study SCEs. The reasons to use Strand-seq rather than classical genetic marker-based SCE assays have been discussed in **Chapters 1 and 4**. In this section, I will only discuss how Strand-seq could be further improved. Moreover, I will also discuss additional applications for Strand-seq, especially with regards to its use to study genome instability during ageing.

Finally, I will close this chapter with few thoughts on how recombination proteins could prevent accelerated senescence.

Models for spontaneous SCEs formation

Elevated levels of SCE are an indication of genome instability, which is a hallmark of both ageing and cancer. However, we still do not know how and why SCEs occur in these cells. It has been estimated that a cell undergo approximately 10,000 lesions per day (Lindahl, 1993). Some of these lesions will be efficiently repair by DNA repair mechanisms prior DNA replication. But if left unrepaired, or if damage occurs during replication, such lesions can affect replication fork passage and lead to stalled or collapsed replication forks. Cells exposed to DNA damaging agents known to affect DNA replication such as methyl methanesulfonate (MMS) show increase

level of SCEs (Perry and Evans, 1975; Morgan and Cleaver, 1982). Therefore, it is possible that spontaneous SCEs come from endogenous replication stress.

The type of DNA damage leading to the formation of SCEs during DNA replication is still unknown. However, studies have shown that DSBs can be repaired by homologous recombination (HR). In the canonical DSB repair (DSBR) pathway, if repair occurs via sister chromatid recombination, a double Holliday junction intermediate that is resolved by structure-specific endonucleases in a manner resulting in a crossover will lead to an SCE (review in Symington et al., 2014). Nonetheless, as detailed in **Chapter 4**, we find that DSBs cannot account for the majority of spontaneous SCEs. A single-strand DNA break (SSB) can also generate an SCE. Deletion of XRCC1 or LIG3, genes involved in base excision repair in mammals, leads to an increase in SCEs (Puebla-Osorio et al., 2006; Thompson et al., 1990). This increase can be either due to the conversion of SSBs to DSBs during replication or a switch in repair mechanisms from base excision repair to DNA damage bypass mechanisms during DNA replication. DNA damage bypass occurs via two main pathways: translesion synthesis (TLS) or error-free post replication repair (PRR). *REV1*, *REV3* and *REV7* are needed for TLS, and deletion of these genes increase SCEs (Simpson and Sale, 2003; Sonoda et al., 2003; Okada et al., 2005), suggesting that in absence of TLS, repair will be mediated via the error-free PRR pathway. Error-free PRR involves the use of the undamaged sister chromatid and therefore, could lead to the formation of SCEs.

HR and error-free PRR pathways are known to use the undamaged sister chromatid as a template for repair. Recently, it has been shown that HR proteins, including Rad51 and Rad52, also act during template switching mechanisms that are thought to be the basis of error-free PRR (Claussin and Chang, 2016; Ball et al., 2009; Gangavarapu et al., 2007). Rad51 initiates HR by mediating strand invasion into double-stranded DNA, leading to the formation of heteroduplex DNA. In the DSBR model, HR continues with the formation of a double Holliday junction intermediate. It is possible that such an intermediate also forms during error-free PRR, although no evidence exists so far. Resolution of the double Holliday junction via a crossover would result in an SCE.

However, we found that spontaneous SCEs were only reduced less than twofold in the absence of Rad51 (**Chapter 4**). Similarly, a previous study using a genetic marker-based assay found that the level of spontaneous SCEs was not changed in yeast lacking Rad51 (Fasullo et al., 2001), indicating that most spontaneous SCEs must occur via a Rad51-independent pathway. Rad51-independent HR has previously been observed, even in the repair of DSBs. In the absence of Rad51, the products of DSB repair are mostly gene conversion, and this is thought to occur by Rad51-independent break-induced replication (BIR) or single strand annealing (SSA) mechanisms (Bai and Symington, 1996; Bartsch et al., 2000; Kang and Symington,

2000; Pohl and Nickoloff, 2008; Signon et al., 2001). Both Rad51-independent BIR and SSA mechanisms require Rad52-mediated strand annealing activity. However, neither BIR nor SSA can generate SCEs. We found that strand-annealing activity is important for the formation of spontaneous SCEs (**Chapter 4**), but we currently do not have a model to explain how this activity could lead to SCEs in the absence of Rad51.

Strand-seq optimization

As discussed extensively in **Chapter 4**, Strand-seq is a powerful new assay to study SCE. However, the requirement of nicking the newly synthesized BrdU-substituted DNA before PCR amplification renders Strand-seq incompatible with whole-genome amplification methods, making Strand-seq very technically challenging. Sequencing of single-cell yeast Strand-seq libraries is particularly difficult given the small genome size and added complication of genomic DNA isolation from cells with a cell wall. Consequently, a significant fraction of yeast Strand-seq libraries fail to provide enough mapped reads to be usable in the analysis of SCE. Considering the costs of library preparation and sequencing, it is imperative to improve the Strand-seq protocol.

Any loss of DNA during library preparation will dramatically reduce the sequencing coverage. Therefore, reducing pipetting steps to avoid loss of genetic material is critical when working with low amounts of DNA. To decrease this loss, we combine both cell lysis and DNA digestion into the same reaction buffer. This step must first begin with the removal of the yeast cell wall, which can only be removed by specific enzymes or strong mechanical shearing (Bzducha-Wrobel et al., 2014; Mann and Jeffery, 1986). In our current protocol, we use zymolyase, a protease that mainly degrades the cell wall by hydrolysis of glucose polymers at the β-1,3-glucan linkages (Kitamura et al., 1971). Treating the cells with zymolyase bears the risk that zymolyase cleaves unspecific substrates (K. Paeschke personal communication). In general, mixtures of zymolyase are hard to control (due to dilution effects in the cell and media). For example, if zymolyase degrades histone proteins, this would affect DNA digestion by micrococcal nuclease (MNase), which is an essential step required for library preparation. Therefore, we are currently exploring alternative protocols that would either remove zymolyase from the digestion buffer or minimize its reaction time.

Additionally, we are working on different options that do not include zymolyase during Strand-seq library preparation. Because removal of zymolyase after sorting is not a realistic option, we are exploring the possibility of using zymolyase prior cell sorting, so instead of sorting cells with intact cell walls, we

would sort spheroplasts. Alternatively, we could use methods to lyse the cell wall without the use of zymolyase, such as by using mechanical shearing methods (e.g. sonication) to break apart the cell wall.

In the current Strand-seq protocol, DNA is fragmented enzymatically with MNase that cuts in between nucleosomes. However, MNase digestion does not yield a homogeneous set of DNA fragments of a precise size. While the majority of the DNA fragments are derived from mononucleosome, some fragments are di-nucleosome in size due to insufficient MNase digestion. Excluding such di-nucleosome fragments will result in a substantial loss of DNA, especially when working with a single yeast cell where every DNA fragment is important. Therefore, expanding the size selection criteria to include di-nucleosome-sized DNA fragment may improve Strand-seq sequencing coverage.

Another reason for loss of DNA during Strand-seq library preparation is the multiple cleanup steps. Reducing these steps will potentially increase the overall quality the library, and this is currently being explored by the Lansdorp lab. Specifically, the current the idea is to combine DNA fragmentation and Illumina adaptor ligation in one step via transposase-catalyzed adaptor insertion, which will significantly reduce the number of pipetting steps and time of library preparation.

Other use for Strand-seq

DSB repair and SCEs

Strand-seq can work in both haploid and diploid cells. In previous **Chapter 4**, we argued that using haploids makes the analysis and recognition of SCEs easier. However, it would be interesting to compare SCEs in haploids and diploids, a feat we can accomplish with yeast. First, it has been observed that the use of the sister chromatid is preferred over the homologous chromosome to prevent mutation or loss of heterozygosity. Here we could take advantage of Strand-seq to quantify and compare spontaneous SCEs formation in both haploid and diploid genome.

Strand-seq could also be used to accurately measure the rate of crossovers versus non-crossovers during the repair of an induced-DSB. Previously, this has only been estimated by analyzing a few recombinants in genetic marker-based assays (Hartwell, 1992; Jackson and Fink, 1981).

Repair of DSBs can also be assessed by combining the modified version of Flp-nick system (Nielsen et al., 2012) and Strand-seq. The Flp-nick system uses the action of the Flp recombinase, which creates DNA nick similar to a type IB topoisomerase (Lee and Jayaram, 1997) at the Flp recognition target site (FRT) (Andrews et al., 1985). During DNA replication, the SSB will be converted into a

DSB.

Quantification of SCEs in different conditions

SCEs can be induced by lesion created by both endogenous and exogenous sources, but aside from DSBs, very little is known about what these lesions could be. Strand-seq could be used to study and compare SCE induced by different DNA damaging agents. For example, we have done a few preliminary experiments with MMS and have found that MMS strongly induces SCE.

There are many possible endogenous sources of DNA damage. We showed that DSBs is not the major source of spontaneous SCEs. We also argue that replication stress could lead to the formation of SCEs. There are many natural barriers to replication fork progression. These include DNA-RNA hybrids, G-quadruplex structures, SSBs, and ribonucleotides incorporated into DNA (Branzei and Foiani, 2010). In yeast, we have the ability to ectopically place such barriers at specific genomic loci. We can then study with Strand-seq how these different replication fork obstacles influence SCE formation.

Other types of recombination can be visualized by Strand-seq

In theory, Strand-seq could be used in yeast for a broad range of research questions. In this thesis, I used Strand-seq for the quantification and mapping of SCEs in WT and recombination mutants. Strand-seq with *mus81*Δ cells has potentially identified another type of recombination event (Figure 1). In this library, we see that one end of chromosome XII did not contain any reads and one end of chromosome XV had reads from both the Watson and Crick strands. In haploids, having reads on both strands in the same genomic location can only result from chromosome fragment duplication, break-induced replication (BIR) or chromosome breakage and missegregation during mitosis (Figure 2). Lack of reads from either strand at one end of chromosome XII could result from telomere addition, BIR or chromosome breakage and loss during mitosis. Deletion of *MUS81* in yeast is known to increase BIR events (Ho et al., 2010), suggesting that the events visualized in this specific library may be due to BIR. However, we currently have too few *mus81*Δ Strand-seq libraries (five) to make any conclusions. Furthermore, it will be much more convincing if we can obtain Strand-seq libraries from both mother and daughter cells from the same cell division. Unlike canonical DNA replication, which is semi-conservative, BIR occurs by conservative DNA synthesis (Donnianni and Symington, 2013; Saini et al., 2013), meaning that if BIR occurs between sister chromatids, one chromatid would contain both parental DNA strands while the other chromatid would have all newly synthesized DNA for the part of the chromosome synthesized by BIR. If we see this reciprocal pattern in

a pair of mother and daughter cells sequenced by Strand-seq, we can be much more confident that these events are the result of BIR.

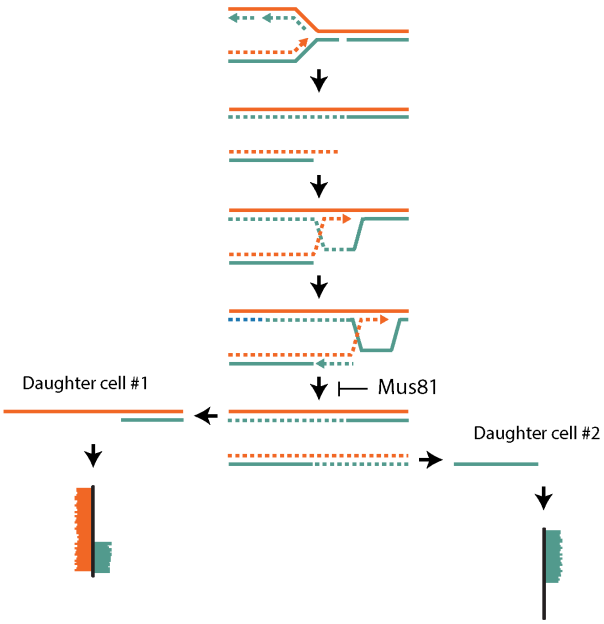

Figure 1. A schematic showing how a BIR event would look by Strand-seq analysis.

Yeast can be micromanipulated on agar plates, which would allow the separation of mother and daughter cells. However, transferring single cells from an agar plate to individual wells of a 96-well plate, suitable for Strand-seq library preparation, may be difficult. Previous studies have shown that DNA can be prepped directly from cells embedded in low-melting point agarose for different applications (Murakami et al., 2009), so the idea should be possible in theory.

Recently, it has been shown that Stran-seq can also be used to visualize polymorphic inversions and de novo haplotyping with human cells (Porubsky et al., 2016; Sanders et al., 2016). Similar experiment regarding inversion could also be done in yeast.

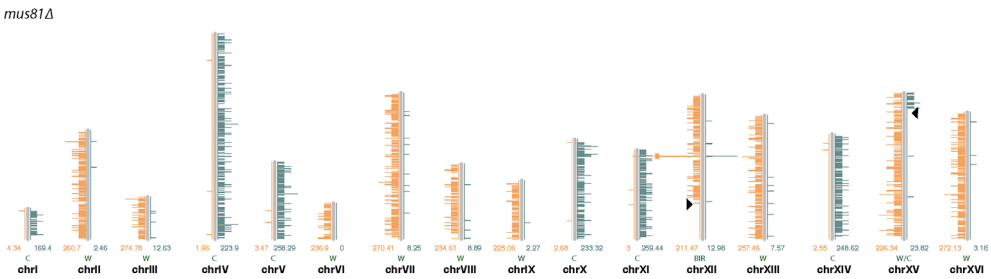

Figure 2. Example of *mus81*Δ Strand-seq library showing potential BIR events.

DNA strand segregation asymmetry hypothesis

If Strand-seq libraries could be prepared from microdissected yeast mother-daughter pairs, then Strand-seq could be also be used to explore whether there is any asymmetry in terms of DNA strand segregation. The immortal strand hypothesis postulates that DNA strands may segregate in a nonrandom manner in certain cell types (e.g. stem cells), retaining a distinct set of template/parental DNA strands in each division to minimize the inheritance of mutations arising during DNA replication (Cairns, 1975). In yeast, this is not occurring in mitotic cells (Keyes et al., 2012). However, such a mode of strand segregation could occur in specific situations. It has been shown that the mother lineage after sporulation exhibits asymmetric segregation of kinetochore proteins, which may be a mechanism for the selective segregation of sister centromeres (Thorpe et al., 2009). Strand-seq could be use to determine if asymmetric DNA strand segregation occurs in the mother spore lineage.

Genome instability in aged-cells

As cancer cells, aged cells are associated with genomic instability (Lopez-Otin et al., 2013). It is not known if the increase in genome instability is due to the accumulation of DNA damage over time or if there is an imbalance between repair pathways during aging. Yeast has been used as a model to study aging. In yeast, three types of aging are commonly studied: telomere-attrition-induced aging (as discussed in **Chapters 2 and 3**), replicative aging and chronological aging. Replicative aging refers to the number of times a mother cell can divide before dying, while chronological aging refers to the progressive decrease in viability of non-dividing cells as a function of time. Replicative ageing can be studied either by micromanipulation of yeast cells by moving the daughter cells away from a mother cell and counting how many

times the mother cell has divided, or by enriching for mother cells from a population of cells using a biotinylation method (Smeal et al., 1996). SCE events could be measured using Strand-seq in different aged cells. Similar experiment employing DNA damaging agents or mutants could also yield to a better understanding of the genome instability during aging.

Recombination at telomeres

In **Chapters 2 and 3**, I have discussed extensively the role of Rad52 in telomere maintenance. In **Chapter 3**, we found we found that impairing the acetylation and deacetylation of H3K56, as well as *rad52* class mutations, causes accelerated senescence in the absence of telomerase (Claussin and Chang, 2016). A similar link between H3K56 acetylation and Rad52-mediated strand annealing was observed with regards to sister chromatid recombination (Munoz-Galvan et al., 2013). This led us to hypothesize that accelerated senescence can be caused by impaired sister chromatid recombination. However, impaired in H3K56 acetylation also causes defects in sister chromatid cohesion (Thaminy et al., 2007). Therefore, sister chromatid cohesion may also be impaired during senescence, which can be experimentally tested in our system.

Concluding remarks

In this thesis, I have focus on understanding sister chromatid recombination (SCR) and its function in the maintenance of genome stability. The ability to detect SCEs with Strand-seq is very useful because elevated levels of SCE is an indication of genome instability, which is a hallmark of both aging and cancer. Moreover, being able to detect SCEs will help us better understand SCR in general. Our work has also highlighted the importance of SCR in telomere biology. Telomere dysfunction is also associated with aging and cancer, so a better understanding of SCR will likely have far-reaching implications for human health.

References

Andrews, B.J., Proteau, G.A., Beatty, L.G., and Sadowski, P.D. (1985). The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. Cell *40*, 795-803.

Bai, Y., and Symington, L.S. (1996). A Rad52 homolog is required for RAD51-independent mitotic recombination in Saccharomyces cerevisiae. Genes & development *10*, 2025-2037.

Ball, L.G., Zhang, K., Cobb, J.A., Boone, C., and Xiao, W. (2009). The yeast Shu complex couples error-free post-replication repair to homologous recombination. Mol Microbiol *73*, 89-102.

Bartsch, S., Kang, L.E., and Symington, L.S. (2000). RAD51 is required for the repair of plasmid double-stranded DNA gaps from either plasmid or chromosomal templates. Molecular and cellular biology *20*, 1194-1205.

Branzei, D., and Foiani, M. (2010). Maintaining genome stability at the replication fork. Nat Rev Mol Cell Biol *11*, 208-219.

Bzducha-Wrobel, A., Blazejak, S., Kawarska, A., Stasiak-Rozanska, L., Gientka, I., and Majewska, E. (2014). Evaluation of the efficiency of different disruption methods on yeast cell wall preparation for beta-glucan isolation. Molecules *19*, 20941-20961.

Cairns, J. (1975). Mutation selection and the natural history of cancer. Nature *255*, 197-200.

Claussin, C., and Chang, M. (2016). Multiple Rad52-Mediated Homology-Directed Repair Mechanisms Are Required to Prevent Telomere Attrition-Induced Senescence in Saccharomyces cerevisiae. PLoS genetics *12*, e1006176.

Donnianni, R.A., and Symington, L.S. (2013). Break-induced replication occurs by conservative DNA synthesis. Proceedings of the National Academy of Sciences of the United States of America *110*, 13475-13480.

Fasullo, M., Giallanza, P., Dong, Z., Cera, C., and Bennett, T. (2001). Saccharomyces cerevisiae rad51 mutants are defective in DNA damage-associated sister chromatid exchanges but exhibit increased rates of homology-directed translocations. Genetics *158*, 959-972.

Gangavarapu, V., Prakash, S., and Prakash, L. (2007). Requirement of *RAD52* group genes for postreplication repair of UV-damaged DNA in *Saccharomyces cerevisiae*. Molecular and cellular biology *27*, 7758-7764.

Hartwell, K.a. (1992). Sister Chromatids Are Preferred over Homologs as Substrates for Recombinational Repair in Saccharomyces Cerevisiae. Genetics *132*, 387-402.

Ho, C.K., Mazon, G., Lam, A.F., and Symington, L.S. (2010). Mus81 and Yen1 promote reciprocal exchange during mitotic recombination to maintain genome integrity in budding yeast. Molecular cell *40*, 988-1000.

Jackson, J.A., and Fink, G.R. (1981). Gene conversion between duplicated genetic elements in yeast. Nature *292*, 306-311.

Kang, L.E., and Symington, L.S. (2000). Aberrant double-strand break repair in rad51 mutants of Saccharomyces cerevisiae. Molecular and cellular biology *20*, 9162-9172.

Keyes, B.E., Sykes, K.D., Remington, C.E., and Burke, D.J. (2012). Sister chromatids segregate at mitosis without mother-daughter bias in Saccharomyces cerevisiae. Genetics *192*, 1553-1557.

Kitamura, K., Kaneko, T., and Yamamoto, Y. (1971). Lysis of viable yeast cells by enzymes of Arthrobacter luteus. Arch Biochem Biophys *145*, 402-404.

Lee, J., and Jayaram, M. (1997). A tetramer of the Flp recombinase silences the trimers within it during resolution of a Holliday junction substrate. Genes & development *11*, 2438-2447.

Lindahl, T. (1993). Instability and decay of the primary structure of DNA. Nature *362*, 709-715.

Lopez-Otin, C., Blasco, M.A., Partridge, L., Serrano, M., and Kroemer, G. (2013). The hallmarks of aging. Cell *153*, 1194-1217.

Mann, W., and Jeffery, J. (1986). Yeasts in molecular biology. Spheroplast preparation with Candida utilis, Schizosaccharomyces pombe and Saccharomyces cerevisiae. Biosci Rep *6*, 597-602.

Morgan, W.F., and Cleaver, J.E. (1982). 3-Aminobenzamide synergistically increases sister-chromatid exchanges in cells exposed to methyl methanesulfonate but not to ultraviolet light. Mutat Res *104*, 361-366.

Munoz-Galvan, S., Jimeno, S., Rothstein, R., and Aguilera, A. (2013). Histone H3K56 acetylation, Rad52, and non-DNA repair factors control double-strand break repair choice with the sister chromatid. PLoS genetics *9*, e1003237.

Murakami, H., Borde, V., Nicolas, A., and Keeney, S. (2009). Gel electrophoresis assays for analyzing DNA double-strand breaks in Saccharomyces cerevisiae at various spatial resolutions. Methods Mol Biol *557*, 117-142.

Nielsen, I., Andersen, A.H., and Bjergbaek, L. (2012). Studying repair of a single protein-bound nick in vivo using the Flp-nick system. Methods Mol Biol *920*, 393-415.

Okada, T., Sonoda, E., Yoshimura, M., Kawano, Y., Saya, H., Kohzaki, M., and Takeda, S. (2005). Multiple roles of vertebrate REV genes in DNA repair and recombination. Molecular and cellular biology *25*, 6103-6111.

Perry, P., and Evans, H.J. (1975). Cytological detection of mutagen-carcinogen exposure by sister chromatid exchange. Nature *258*, 121-125.

Pohl, T.J., and Nickoloff, J.A. (2008). Rad51-independent interchromosomal double-strand break repair by gene conversion requires Rad52 but not Rad55, Rad57, or Dmc1. Molecular and cellular biology *28*, 897-906.

Porubsky, D., Sanders, A.D., van Wietmarschen, N., Falconer, E., Hills, M., Spierings, D.C., Bevova, M.R., Guryev, V., and Lansdorp, P.M. (2016). Direct chromosome-length haplotyping by single-cell sequencing. Genome research *26*, 1565-1574.

Puebla-Osorio, N., Lacey, D.B., Alt, F.W., and Zhu, C. (2006). Early embryonic lethality due to targeted inactivation of DNA ligase III. Molecular and cellular biology *26*, 3935-3941.

Saini, N., Ramakrishnan, S., Elango, R., Ayyar, S., Zhang, Y., Deem, A., Ira, G., Haber, J.E., Lobachev, K.S., and Malkova, A. (2013). Migrating bubble during break-induced replication drives conservative DNA synthesis. Nature *502*, 389-392.

Sanders, A.D., Hills, M., Porubsky, D., Guryev, V., Falconer, E., and Lansdorp, P.M. (2016). Characterizing polymorphic inversions in human genomes by single-cell sequencing. Genome research *26*, 1575-1587.

Signon, L., Malkova, A., Naylor, M.L., Klein, H., and Haber, J.E. (2001). Genetic requirements for RAD51- and RAD54-independent break-induced replication repair of a chromosomal double-strand break. Molecular and cellular biology *21*, 2048-2056.

Simpson, L.J., and Sale, J.E. (2003). Rev1 is essential for DNA damage tolerance and non-templated immunoglobulin gene mutation in a vertebrate cell line. The EMBO journal *22*, 1654-1664.

Smeal, T., Claus, J., Kennedy, B., Cole, F., and Guarente, L. (1996). Loss of transcriptional silencing causes sterility in old mother cells of S. cerevisiae. Cell *84*, 633-642.

Sonoda, E., Okada, T., Zhao, G.Y., Tateishi, S., Araki, K., Yamaizumi, M., Yagi, T., Verkaik, N.S., van Gent, D.C., Takata, M., *et al.* (2003). Multiple roles of Rev3, the catalytic subunit of polzeta in maintaining genome stability in vertebrates. The EMBO journal *22*, 3188-3197.

Symington, L.S., Rothstein, R., and Lisby, M. (2014). Mechanisms and Regulation of Mitotic Recombination in *Saccharomyces cerevisiae*. Genetics *198*, 795-835.

Thaminy, S., Newcomb, B., Kim, J., Gatbonton, T., Foss, E., Simon, J., and Bedalov, A. (2007). Hst3 is regulated by Mec1-dependent proteolysis and controls the S phase checkpoint and sister chromatid cohesion by deacetylating histone H3 at lysine 56. The Journal of biological chemistry *282*, 37805-37814.

Thompson, L.H., Brookman, K.W., Jones, N.J., Allen, S.A., and Carrano, A.V. (1990). Molecular cloning of the human XRCC1 gene, which corrects defective DNA strand break repair and sister chromatid exchange. Molecular and cellular biology *10*, 6160-6171.

Thorpe, P.H., Bruno, J., and Rothstein, R. (2009). Kinetochore asymmetry defines a single yeast lineage. Proceedings of the National Academy of Sciences of the United States of America *106*, 6673-6678.